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(54) Title: NOVEL COMPONENT OF AMYLOID IN ALZHEIMER'S DISEASE AND METHODS FOR USE OF SAME

(57) Abstract

A gene, NACP, is disclosed along with its nucleotide and amino acid sequence. Also disclosed are diagnostic and therapeutic methods of utilizing the NACP nucleotide and polypeptide sequences. The NACP polypeptide is a precursor of NAC, a peptide associated with amyloid deposits in the brains of patients with typical neuropathological features of Alzheimer's disease (AD). Also disclosed is the amino acid sequence of NAC and of two contiguous fragments thereof, X and Y peptides. Diagnostic and therapeutic methods relating to amyloid disorders associated with NAC are also disclosed.

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NOVEL COMPONENT OF AMYLOID IN ALZHEIMER'S DISEASE AND METHODS FOR USE OF SAME

STATEMENT OF GOVERNMENT SUPPORT

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RELATED U.S. PATENT APPLICATIONS

This application is a continuation in part of U.S. patent application serial no. 08/114,393, filed on August 30, 1993.

BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates to diagnosis and treatment of neuronal abnormalities, in particular the deposition of amyloid plaques characteristic of Alzheimer's Disease.

Description of Related Art

The most common cause of disabling dementia in humans is Alzheimer's disease ("AD"). Its incidence increases sharply with age, and it is a major public health problem in our aging population. Persons suffering from Alzheimer's disease show a characteristic neuropathology, including synaptic loss, senile plaques and neurofibrillary tangles. Neurofibrillary tangles comprise paired helical filaments ("PHF") (D.L. Selkoe, *et al.*, *Science*, 235:873-876,

1987). A senile plaque commonly comprises a mass of disorganized neurites surrounding a deposit of extracellular filaments of an amyloid polypeptide called A4 or β amyloid protein ("Aβ").

Deposition of fibrillar deposits of Aβ a 39/43 residue amyloid, is considered the pathological hallmark of AD. Recently, molecular cloning based on the sequence of the Aβ protein indicated that it is encoded as part of a larger precursor (PreA4) that maps to chromosome 21 (Kang, *et al.*, *Nature*, 325:733-736, 1987; Goldgaher, *et al.*, *Science*, 235:877-880, 1987; Tanzi, *et al.*, *Science*, 235:880-884, 1987; Robakis, *et al.*, *Proc. Natl. Acad. Sci.* 84:4190-4194, 1987). There are three major alternatively spliced products of the amyloid mRNA (Ponte, *et al.*, *Nature*, 331:525-527, 1988,; Tanzi, *et al.*, *Nature*, 331:528-530, 1988; Kitaguchi, *et al.*, *Nature*, 331:530-532, 1988). The smallest of these products, the 695-residue precursor protein ($PreA4_{695}$), has been synthesized *in vitro* and shown to be a N-glycan membrane protein that spans the lipid bilayer once (Dyrks, *et al.*, *EMBO J.*, 7:949-957, 1988). Two other forms of PreA4 (PreA4 751 and PreA4 770) contain a 56 residue insert which has a protease-inhibitory function. The amyloidogenic A4 protein is derived in part from the transmembrane domain and from part of the adjacent extracellular domain. A precursor-product relationship has been demonstrated.

The A4 gene is expressed in brain and peripheral tissues, such as muscle and epithelial cells (Goeder, *EMBO J.*, 6:3627-3632, 1987; Bahmanyar, *et al.*, *Science*, 237:77-88, 1987; Zimmerman, *et al.*, *EMBO J.*, 7:1365-1370, 1988; Shivers, *et al.*, *EMBO J.*, 7:1365-1370, 1988), yet for reasons still unknown, the amyloid deposits in AD are confined to the brain.

Recently, *in situ* hybridization analyses were published that indicate an alteration of the amount of PreA4 mRNA in brains of AD patients when compared to normal individuals (Higgins, *et al.*, *Proc. Nat'l Acad. Sci. USA*, 85:1297-1301, 1988; Cohen, *et al.*, *Science*, 237:77-88, 1987; Lewis, *et al.*, *Proc. Nat'l Acad. Sci. USA*, 85:1691-169, 1988). These results implicate a role for gene regulation in AD.

In addition to Aβ, heparan sulfate proteoglycan, ferritin, immunoglobulins, and many acute phase proteins such as α-1 antichymotrypsin, apolipoprotein E, complements, serum amyloid P, and trace peptides have been reported to be associated with amyloid. However, supportive biochemical data demonstrating the presence of these proteins in amyloid preparations from the brains of Alzheimer's victims are not yet available, raising the possibility that these may not be intrinsic components of amyloid.

All forms of amyloid in amyloid deposits, including the Aβ, show a significant β-pleated sheet component (Snow, A.D., *et al.*, 1987). Yet the precursor of amyloid Aβ protein is soluble and does not exhibit a significant β-pleated sheet component. Recent studies of C. Haass, *et al.*, *Nature*, 359:322-325, (1992); P. Seubert, *et al.*, *Nature*, 359:325-327 (1992); M. Shoji, *et al.*, *Science*, 258:126-129 (1992), have demonstrated that Aβ is generated and secreted from various types of cells under physiological conditions, implying that Aβ is soluble in aqueous solutions.

The physiological process responsible for changing the structure of the precursor protein is the subject of much enquiry. Recently a study by Roses, *et al.*, demonstrates that apolipoprotein E binds Aβ (*Proc. Natl. Acad. Sci. USA*, 90:1977-1981, 1993). Thus, apolipoprotein E may act as a molecular chaperone that mediates the β-pleated amyloid formation of Aβ as suggested by T. Wisniewski, *et al.* (*Neurosci. Lett.*, 135:235-238, 1992).

New and further information concerning the molecular biology involved in formation of amyloid deposits such as those found in Alzheimer's disease awaits discovery of additional intrinsic constituents associated with amyloid in the brains of those affected with Alzheimer's disease. On a physiological basis, recent studies have shown that amyloid deposition might be the result of aberrant processing of APP, and its abundance is an important parameter to consider in diagnosing the disease on a neuropathological basis. In addition, the cognitive dysfunction that characterizes AD is apparently attributable to synaptic loss (Terry, *et al.*, *Ann.Neurol.*, 30:572-580 (1993); Mattson, *et al.*, *TINS*, 16:409-414 (1993)). Recent studies strongly suggetsthat there is a connection between the abnormal processing of synaptic proteins and amyloid formation (Masliah, *et al.*, *Brain Path.*, 3:77-85 (1993)).

However, despite the knowledge that AD is related to neuritic plaques and synaptic loss, diagnosis of the disease is difficult. Currently, the only way of confirming the presence of these lesions in a living patient is by brain biopsy. However, this technique is rarely utilized because of the substantial risks to the patient involved in performing it. As a result, AD is usually diagnosed on the basis of clinical symptoms and the results of neuropsychological tests. Nonetheless, because AD can be mimicked by other disorders (such as

depression), confirmation of an AD diagnosis often cannot be conclusively made until autopsy.

Several methods for *in vivo* diagnosis of AD have been proposed but have not yet yielded definitive results. One such approach attempts to detect amyloid and/or the precursor protein for it (APP) in blood and cerebrospinal fluid. These measurements have not, however, been shown to positively correlate to the development of neuritic plaques in AD. Another approach involves detection of a mutated form of the gene for the amyloid precursor protein. While the presence of this genetic alteration appears to be more predictive of AD than does circulating levels of amyloid and APP, the mutated gene is only found in some familial cases of AD. As a result, presence of the mutated gene would correlate to the onset of AD in less than 1% of all potential AD cases.

In vivo diagnosis of AD is further limited by the blood/brain barrier. Because of the barrier, detection of amyloid deposits by binding assays (and evaluation of synaptic loss associated with dementia) has been limited to autopsy studies (see, e.g., Masliah, *et al., Am. J. Pathol.*, 137:1293-1297, 1990 [quantitation of synapse loss in brain tissue section through use of labelled anti-synaptophysin antibodies]).

Further, the blood/brain barrier has also prevented (to date) the effective use of antibodies for *in vivo* diagnosis and therapy of AD. Thus, a promising *in vitro* use of a monoclonal antibody 10H3 which targets amyloid deposits (Majocha, *et al. J. Nucl. Med.*, 33:2184-2189) has not yet been extended to an *in vivo* application. Due to the size of antibodies like 10H3, there is some doubt whether they can successfully and innocuously cross the blood/brain barrier.

Clearly, a need exists for a noninvasive method for *in vivo* detection of amyloid deposits in brain tissue of patients who are suspected of having AD. In combination with present techniques for clinical diagnosis of AD, such a technique would be useful in confirming a diagnosis of, and evaluating the prognosis for, the disease.

SUMMARY OF THE INVENTION

Novel peptides obtained by purification and analysis of the amino acid sequences in an amyloid preparation of brain tissue obtained from patients with symptoms of Alzheimer's disease have been shown to be fragments of a novel amyloid component ("NAC"). Antibodies were raised against synthetically produced fragments of these novel peptides and used in immunohistochemical and electron microscopic analyses demonstrating that the peptides are localized in amyloid fibrils in AD brain tissue and are amyloidogenic.

Complementary DNA ("cDNA") encoding a 140 amino acid protein identified as the precursor ("NACP") of NAC is provided. NACP is a highly abundant synaptic protein, which degrades to form NAC. NAC is self-aggregating; i.e., it has a significant ability to bind to itself and become part of amyloid fibrils and neuritic plaque. The invention therefore provides NAC and NACP peptides useful as ligands to identify and quantify syanpses and plaques toward diagnosis and monitoring of diseases associated with synaptic loss and neuritic plaque formation, such as AD. The invention also provides methods for treatment of such diseases.

In particular, in the preferred diagnostic embodiment of the invention, a detectably labelled NAC/NACP peptide which will specifically bind NAC deposits in brain tissue, is administered parenterally to a mammal (preferably a human). Binding of the administered peptide to NAC/NACP in brain tissue is detected using suitable *in vivo* diagnostic imaging techniques. Most preferably, this detection will be by positron emission tomography (PET) or single photon emission computed tomography (SPECT).

In another aspect of the invention, the binding data generated as described above is evaluated with any clinical signs of a neuropsychological disorder to assist in confirmation or refutation of an initial diagnosis of AD. To the extent that the binding data reveals the extent of amyloid deposition, the data may also be used to evaluate the prognosis for a patient with a confirmed diagnosis of AD. Thus, the diagnostic method of the invention will provide physicians with valuable information concerning the medical status of a patient who is suspected of suffering from AD.

In another aspect of the invention, the binding data described above is evaluated with the results of tests for synapse loss in brain tissue to assist in confirmation of an AD diagnosis and to evaluate the prognosis for the patient. Data evidencing a relationship between amyloid deposition and synapse loss in AD brain tissue will also be of use in research toward understanding the etiology of AD.

In another aspect of the invention, labelled NAC/NACP peptides are utilized in *in vitro* studies of amyloid deposition in sections of brain tissue for use in confirming an AD diagnosis and/or for research purposes. For example, *in vitro* (and *in vivo*) use of detectably labelled NAC/NACP peptides may be used to evaluate agents to inhibit NAC formation, binding and deposition.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 A shows immunohistochemical staining with anti-NAC antibodies of amyloid in diffuse, primitive, and mature plaques on slides of brain sections from patients with symptoms of Alzheimer's disease. In Panel A hippocampal sections were stained with anti-NAC antibody, anti-X1. In Panel B, in addition to amyloid staining, occasional staining of dystrophic neurites (arrows) was detected with a anti-NAC antibody, anti-Y. Absorption with the corresponding peptide eliminated the staining. Panel C shows absence of immunohistochemical staining by anti-sera to NAC on slides of AD brain sections when pre-absorbed with NAC peptides. Panel D shows an electron micrograph of specific staining by anti-X1 antibody on amyloid fibrils (arrows) in AD brain sections. Amyloid fibrils were also stained with anti-Y antibody.

Figure 2 A shows the nucleotide sequence of cDNA encoding the precursor of the NAC protein and the 140 amino acid sequence of the NAC precursor protein encoded by a 420 bp open reading frame with the X and Y fragments located contiguously in the middle of the precursor. The nearest in-frame stop codon (TAA) upstream to the putative initiation methionine codon is marked by an asterisk. The termination codon is marked by two asterisks. Sequence for X and Y peptides are boxed. Synthetic oligonucleotide mixtures used for PCR are indicated as lines above the corresponding cDNA. Polyadenylation signals are underlined.

Figure 2 B is a graph showing the hydropathy profile of the NAC precursor protein with the NAC region being the most hydrophobic.

Figure 3 is a Western blot of brain homogenate with anti-NAC polyclonal antibody anti-X1. NAC precursor protein in lane 4 is detected as a M_r 19K protein. The bacterially expressed NAC precursor protein expressed in *E. coli* transfected with pSENACP migrated in lanes 2 and 5. Lanes 1 and 6 contain *E. coli* transfected with pSE380 vector control; and lanes 3 and 4 show normal human brain. Lanes marked with (+) indicate X1 antibody was preabsorbed with X1 peptide; while those marked (-) indicated X1 antibody was preabsorbed with a control peptide. The arrow indicates NACP detected as a M_r 19K protein whose staining was blocked by preabsorption of the antibody with X1 peptide, thus showing specificity of the antibody.

Figure 4 A is a sequence listing of seven repeated sequence motifs in the NAC precursor amino acid sequence.

Figure 4 B is a sequence listing showing homology in the NAC precursor amino acid sequence at amino acids 48-56 and 70-78. Bold letters indicate the common amino acids among the repeat.

Figure 4 C is a comparison of the cDNA listings of EST01420 (EMBL/GenBank Libraries) and the NAC precursor showing homology therebetween at the N-terminal region of NAC.

Figure 5 is a Northern Blot of mRNA for NACP. Lane 1 shows normal adult midfrontal cortex (female, aged 88); lane 2 shows cerebellum from the same individual as lane 1; lane 3 shows fetal whole brain (female, 24 week fetus); lane 4 shows midfrontal cortex from individual with AD (female, aged 83); lane 5 shows cerebellum from the same individual as Lane 4; lane 6 shows normal adult liver (male, aged 18); lane 7 shows normal child lung (male, aged 7).

Figure 6 A depicts self-aggregation of NAC peptide at various times and peptide concentrations (10-300 μM) as measured by turbidity at OD 400 nm. The NAC aggregation was studied in PBS (pH 7.4) solution at 37°C.

Figure 6 B depicts self-aggregation of NAC peptide (concentration, 300 μM) at various times and temperatures (4°, 22°, and 37°C) as measured by turbidity at OD 400 nm. The NAC aggregation was studied in PBS (pH 7.4) solution.

Figure 7 is a Western blot analysis of NAC peptide aggregation. A NAC peptide monomer migrated at an apparent molecular mass of the 3500 Da. The signal intensity of 3500 Da band was significantly decreased on Days 5 and 7. On the other hand, aggregated NAC peptide was found at the top of the gel. This signal increased to a maximum by Day 3. No intermediate-size bands were observed.

Figures 8A and B show birefringence of Congo red-stained NAC peptide viewed by cross-polarization microscopy. Bright-field (A) and cross-polarized light (B) pictures of NAC peptide preparation stained with Congo red are shown.

Figure 9 is an electron micrograph of aggregated NAC peptide.

Figures 10 A through D show (both macro- and microscopically) immunostaining of NACP in rat brain using anti-NACP(131-140) with streptavidin-biotin-peroxidase (SAB) method. In FIGURE 10 A is a macroscopic image of stained sagittal brain section showing that NACP immunoreactivity was relatively strong in the neocortex, olfactory region, caudoputamen,

hippocampus, and cerebellar cortex compared to the thalamus and brain stem. Higher magnification microscopic analysis showed a characteristic granular immunoreactivity throughout the brain. In FIGURE 10 B is a microscopic image of stained cerebellar cortex. In FIGURE 10 C is a microscopic image of stained hippocampal dentate gyrus. In FIGURE 10 D is a microscopic image of stained cerebral cortex.

Figures 11 A through I are photographs obtained by laser scanning confocal microscopy of sections double-labeled for SY38 (mouse monoclonal anti-synaptophysin antibody; labeled with FITC and shown by bright patches in panels A, D and G) and NACP(131-140/SEQ.ID.No.6; shown by bright patches in panels B, E and H). The right hand panels (C, F and I) correspond to the electronically merged image; colocalization of NACP with synaptophysin is indicated by bright patches. Panels A-C are derived from staining of neocortex tissue; panels D-F are derived from staining of glomeruli of the olfactory bulb; and, panels G-I are derived from staining of the cerebellar cortex. The scale bar (a horizontal white line across the lower right hand corner of panel A) is equal to 15 μm.

Figure 12 is a bar graph developed by computer-aided quantification of colocalization of NACP with synaptophysin in the presynaptic terminals of rat brain tissue. In the cortical regions a large percentage of the synpatophysin-immunoreactive terminals contained NACP. In contrast, in subcortical regions, a lower proportion of the synapatophysin-immunoalabeled axosomatic nerve terminals contained NACP.

Figure 13 is a photograph obtained by immunoelectron microscopy which reveals the synaptic vesicle membrane localization of NACP. Vibratome sections were immunostained with anti-NACP(131-140) and analyzed by electron microscopy.

Figure 14 is a Western blot quantifying NACP, APP, and synaptophysin in rat brain sections. Immunostaining of NACP, APP, and synaptophysin bands was carried out using anti-NACP(131-140), mouse monoclonal antibody, 22C11, and mouse monoclonal antibody, SY38, respectively. Signal intensity was quantified by scanning by a densitometer. Each value is shown as relative amount of protein normalized to the value in frontal cortex. NACP is highly concentrated in olfactory bulb, frontal cortex, striatum, and hippocampus , whereas APP and synaptophysin are distributed uniformly throughout the brain.

Figure 15 A-B shows, in bar graph form, the number of NACP containing and synaptophysin containing synaptic terminals present per 100 sq/μm of human frontal cortex brain tissue.

Figure 15 C-D shows, in bar graph form, the pixel intensity detected per synapse of human frontal cortex brain tissue indicative of the average quantities of NACP and synaptophysin contained in each synapse. The solid bars are indicative of the values obtained in brain tissue from persons without AD; the slashed bars are indicative of the values obtained in brain tissue from persons suffering from AD.

Figure 16 shows immunolabeling of NACP and synaptophysin in human brain tissue obtained from a healthy person (upper panels) and from a person suffering from AD (middle panels). The left and right images from the middle panel are electronically merged in the lower panel. Areas showing the brightest in the lower panel indicate colocalization of NACP and synaptophysin.

Figure 17 shows immunolabeling of NACP and synaptophysin in mature plaques (the 2 upper left hand panels) and in diffuse plaques (the 2 upper right hand panels). The left and right images from the upper panels are electronically merged in the lower panel. Areas showing the brightest in the lower panel indicate colocalization of NACP and synaptophysin.

Figure 18 shows immunolabeling of β-amyloid (left hand panels) and NAC (right hand panels, with results electronically superimposed on the left hand panels) in brain tissue from healthy, elderly persons (panels A and B), from persons suffering from the early stages of AD (panels C and D), and from persons suffering from advanced stages of AD.

DETAILED DESCRIPTION OF THE INVENTION

A. FUNCTIONAL AND STRUCTURAL CHARACTERISTICS OF NAC AND NACP.

The present invention provides a novel amyloid component (NAC). As used herein "NAC" shall mean Non-Aβ component of AD amyloid. "Aβ" as used herein shall mean fibrillar deposits of the A4 protein, a 39/43 residue amyloid. "AD" as used herein shall mean Alzheimer's Disease. "NAC associated amyloid disorder" shall refer to diseases associated with the excessive formation of amyloid in brain tissue, concommitant synaptic loss, and related cognitive dysfunction. This novel component of amyloid was discovered by analysis of the entire amino-acid sequences in an amyloid preparation of the frontal cortex of patients with typical neuropathological features of Alzheimer's disease (AD) using methods of purification in SDS and sequencing well known in the art. Hence, NAC is the second intrinsic component after Aβ to be found in AD amyloid.

NAC, which is expressed as a larger precursor polypeptide NACP, was found by both biochemical and immunohistochemical evidence to be an intrinsic component of amyloid in AD brain tissue. Copurification of NAC with amyloid in the presence of SDS and immunological localization on amyloid fibrils at the electron microscopic level shows that NAC is localized in neuritic plaques and amyloid fibrils.

As shown in FIGURE 2, NACP is encoded by a 1560 base pair polynucleotide (SEQ. I.D. NO. 1) with a 420 bp open reading frame which encodes a 140 amino acid polypeptide NACP (SEQ. I.D. NO. 2) that is the precursor of NAC, a polypeptide of at least 35 amino acids in length (SEQ. I.D. NO. 3). *In vivo*, NACP is recovered in the cytosolic fraction of human brain homogenate as a protein with an apparent molecular mass of 19,000 Da.. NACP has seven repeated KTKEGV amino acid motifs, but no signal petide sequence nor N-linked glyclosylation sites. NAC is located in the most hydrophobic portion of NACP. NAC is at least 35 amino acids and has a molecular weight of approximately Mr 3,500. Within NAC, two new amyloid sequences have been identified and known herein as the "X and Y peptides", which are encoded contiguously in the most hydrophobic domain (SEQ. I.D. NOS. 4 and 5, respectively). The definite length of NAC was not determined due to the use of enzymatic digestion in its preparation.

The association of NAC in amyloid deposits in AD brain tissue differs from that of both Aβ and α1-antichimotrypsin (ATC), two proteins generally used as indicators of the presence of amyloid in Alzheimer's Disease. Recent work has shown that 50% of intracellular neurofibrillary tangles (NFT) and 100% of extracellular NFT contain Aβ (G. Perry, *et al.*, *Am. J. Pathol.*, 140:283-290, 1992). Immunohistochemical studies of the distribution of NAC in AD brain tissue (See Example 2 herein) found that NAC was not present in NFTs.

The association of NAC with amyloid in the brains of patients with the symptoms of Alzheimer's Disease (AD) is high. Although ATC has been reported to be localized on amyloid fibrils in brain tissue at the electron microscopic level (C. R. Abraham, *et al.*, *Cell*, 52:487-501, 1988), biochemical analysis of amyloid AD brain tissue prepared and analyzed as in Examples 1-3

below, revealed no ATC. This result suggests that the amount of ATC in amyloid may be too small to detect in the present preparation, or, alternatively, that the association of ATC with amyloid may be less significant than that of NAC and, therefore, ATC may be lost during preparation of amyloid used herein.

Thus, as an indicator of the deposition of amyloid in brain tissue, NAC is both more specific to neuritic plaque and amyloid fibrils than ATC and less likely to be lost in preparation of tissues to be tested than ATC.

NAC is strongly hydrophobic and has characteristics associated with a tendency to form a β-pleated secondary protein structure. When synthesized chemically, NAC aggregates and precipitates easily in aqueous solution in a time, concentration and temperature-dependent manner. More particularly, synthetic NAC was detected initially as a monomer of 3500 Da, but became aggregated in aqeuous solution into a higher molecular weight molecule that could not migrate into an electrophoretic gel. On Congo red staining, the NAC aggregate showed green-gold birefringence when viewed with a poloarized light microscope and had a fiber-like structure when viewed through an electron microscope.

Based on the relative yield of peptides X, Y and Aβ sequences in amyloid preparations, the concentration of NAC in amyloid seems to be less than ten percent that of Aβ. Further, double-immunostaining of NAC with β-amyloid antibodies revealed that NAC is more abundant in mature than in diffuse plaques. Interestingly, diffuse plaques from "normal" control tissue do not react with anti-NAC, whereas early and advanced AD cases cotaining large numbers of diffuse and/or at least some mature plaques display relatively strong anti-

NAC reactivity (in approximately 30-50% of the plaques) (see, FIGURE 18). These data suggest that (1) there is a connection between metabolism of presynatpic proteins and plaque formation, and (2) NAC follows diffuse β-amyloid accumulation into mature plaques.

In contrast, it does not appear that NACP is substantially present, if at all, in amyloid. For example, no other sequences of NACP besides the X and Y peptides were detected in the peaks eluted from HPLC analysis of the NACP protein. Further, while NAC was identifed in immunostained amyloid on Western or dot blot, NACP was not. Thus, it appears that NAC can form amyloid *in vivo* after cleavage from its precursor (NACP) and is likely to play a substantial role in amyloidosis. However, because the amyloid found in the brain tissue of humans with confirmed diagnoses of AD differs in structure from NAC aggregates alone, it is likely that NAC is not the sole component of amyloid. Rather, it is most probable that NAC is involved in the initial stages of amyloid formation, leaving the principal development of amyloidosis to the accumulation of β-amyloid.

It should be appreciated, however, that observations have been made that proteins that bind to β-amyloid retard its accumulation (see, e.g., Strittmatter, *et al.*, *Proc.Natl.Acad.Sci. USA*, 90:8098-8102 (1993); Fraser, *et al.*, *J.Neurochem.*, 61:298-305 (1993); and, Schwarzman, *et al.*, *Ann.N.Y.Acad.Sci.*, 6:139-143 (1993)). Thus, with the knowledge of NAC's role in amyloidosis set forth herein, it can be reasonably expected that binding of NAC by NAC polypeptides will retard its accumulation as well, thereby slowing the progression of disease associated with amyloid plaque formation.

With respect to NACP, immunostaining indicates that it, like the APP precursor of β-amyloid, is a presynaptic protein. Specifically, using the streptavidin-biotin-peroxidase staining method, rat brain sections were immunostained by an antibody raised to the NACP amino acid sequence from position 131-140 (see, SEQ.ID.No.2) and position 1-9 (*id.*). Throughout the various cortical and subcortical areas of the rat brain, anti-NACP (131-140) and (1-9) immunostained the neuropil in a characteristic punctate pattern. Neuronal cell bodies, glial cells and blood vessels were not immunostained.

NACP does not have a signal sequence, which suggests that NACP proteins remain localized in neuronal cytoplasm where NACP is expressed. However, it has been discovered that NAC can seep out of cells under certain conditions, such as serum deprivation. Further, as shown in FIGURE 4A, the NACP protein is characterized by repetitive motifs. The KTKEGV motif is repeated seven times, but the amino acid positions 2 to 6 are sometimes substituted. In addition, as shown in FIGURE 4B, amino acids 48-56 and 70-78 of the NACP protein are homologous. These repeated motifs can prove useful in determining the secondary and tertiary protein structure as well as the biological function and metabolism of this protein.

For instance, in accordance with the teachings of P. J. Kennelly, *et al.* (*J. Biol. Chem.*, 256:15555-15558, 1991), the threonine residues in the KTKEGV motif would offer favorable targets for protein kinase C (PKC). The action of this enzyme is known to be critical in determining the functional state of neurons (Y. Nishizuka, *Nature*, 334:661-665, 1988).

In addition, laser scanning confocal microscopic analysis of sections double immunolabeled with antibodies against NACP and synaptophysin (a synaptic

vesicle protein; see, e.g., Masliah, *et al., J.Neurosci.*, 11:2759-2767 (1991)) showed that both markers colocalized in the great majority of the presynaptic terminals, and that NACP is colocalized with synaptophysin in approximately 80% of the presynaptic boutons and in the neuritic component of plaque (FIGURES 11 through 17). Ultrastructural analysis of sections immunolabeled with NACP confirmed the synpatic localization of this protien and showned that NACP was associated with the synaptic vesicles (FIGURE 13). As compared to synaptophysin and APP (which are distributed fairly evenly throughout the brain), NACP was concentrated in the telecephalon, suggesting a functional role for NACP in that region of the brain (FIGURE 14).

As shown in FIGURE 3, NACP is detected in the cytosolic fraction of brain homogenates and comigrates on Western blots with NACP synthesized in *E. coli* from NACP cDNA. NACP was not detected in a particulate fraction from human cortex or from NACP-expressing *E. coli*. NACP mRNA is expressed principally in the brain, but is also expressed in low concentration in all tissues examined except in liver, suggesting that it has ubiquitous functions as well as brain specific functions.

Interestingly, in AD brain, the total population of NACP-containing presynaptic terminals is significantly diminished (by 30-40% see, FIGURE 15) as compared to "normal" brain tissue; i.e., brain tissue without a diagnostically significant quantity of plaque (defined further below). At the same time, although the total population of such terminals is decreased in the AD brain, the concentration of NACP in each remaining presynaptic bouton, indicating a mechanism to compensate for the overall level of NACP-expressing terminals.

As further evidence of NACP as a synaptic protein, search of the EMBL/GenBank DNA sequence databases reveals homologies between NACP and rat brain synucleins, the electric organ synpase of *Torpedo califomica* (Pacific electric ray) and to bovine phosphoneuroprotein 14, a brain specific protein present in synapses around neurons but not in glial cells and Purkinje cell bodies. This group of small, acidic, brain-specific proteins have common repetitive sequence motifs and similar hydrophobic profiles (see, Maroteaux, *et al., Mol.Brain Res.*, 11:335-343 (1991); Maroteaux, *et al., J.Neurosci.*, 8:2804-2815 (1988); and, Nakajo, *et al., Eur.J.Biochem.*, 217:1057-1063 (1993)).

In addition, according to the GenBank database, homology exists between NACP and EST01420, a human 223 bp sequence recently identified by random sequencing of human brain cDNA (M. D. Adams, *et al., Nature*, 355:632-634, 1992). Comparison of the sequence of these proteins expressed in the human brain, as shown in FIGURE 4C, indicates the two proteins are substantially homologous in the N-terminal region, but the EST01420 sequence has the termination codon at base pair position 206, and, therefore, could encode only 51 amino acids.

NACP therefore appears to be a member of a family of synaptic proteins having hydrophobic regions centered in an otherwise hydrophilic molecule.

B. NAC/NACP POLYNUCLEOTIDES AND POLYPEPTIDES.

The term "substantially pure" means any NAC or NACP polypeptide of the present invention, or any gene encoding a NAC or NACP polypeptide, which is essentially free of other polypeptides or genes, respectively, or of other contaminants with which it might normally be found in nature, and as such exists in a form not found in nature.

As used herein, the term "functional polypeptide" refers to a polypeptide which possesses a biological function or activity that is identified through a defined functional assay and which is associated with a particular biologic, morphologic or phenotypic alteration in the cell. The biological function can vary from a polypeptide fragment as small as an epitope to which an antibody molecule can bind to as large as a polypeptide which is capable of participating in the characteristic induction or programming of phenotypic changes within a cell. A "functional polynucleotide" denotes a polynucleotide which encodes a functional polypeptide as described herein.

For example, preferred NAC/NACP polypeptides of the invention will be those which will effectively cross the blood/brain barrier without toxic effect. NAC polypeptides of the invention will specifically bind NAC *in vivo*; the peptides will, therefore, have at least one binding site for NAC.

Further, the NAC/NACP polypeptides should not be pathogenic or immunogenic. To the former end, the peptides are soluble and, in the case of NAC peptides, will reversibly bind NAC. To the latter end, the polypeptides are preferably purified from a human or will be synthesized. "Synthesized" in this context refers to peptides produced through human intervention, whether by

chemical synthesis, recombinant genetic techniques or modification of an isolated native peptide.

It should be noted that NAC/NACP polypeptides used in the method of the invention may differ in amino acid sequence or structure but still retain the same biological activity as described above. Such modifications may be deliberately made (by, for example, site-directed mutagenesis) or may occur spontaneously. In either case, the invention will encompass the use of NAC/NACP peptides which have the same phenotype regardless of differences in structure and length between the peptides. These phenotypically similar peptides will be considered to "substantially similar" to one another.

On the molecular level, a molecule is said to be "substantially similar" to another molecule if the sequence of amino acids in both molecules is substantially the same. Substantially similar amino acid molecules will possess a similar biological activity. Thus, provided that two molecules possess a similar activity, they are considered variants as that term is used herein even if one of the molecules contains additional amino acid residues not found in the other, or if the sequence of amino acid residues is not identical. As used herein, a molecule is said to be a "chemical derivative" of another molecule when it contains additional chemical moieties not normally a part of the molecule. Such moieties may improve the molecule's solubility, absorption, biological half life, etc. The moieties may alternatively decrease the toxicity of the molecule, eliminate or attenuate any undesirable side effect of the molecule, etc. Moieties capable of mediating such effects are disclosed, for example, in *Remington's Pharmaceutical Sciences*, 16th ed., Mack Publishing Co., Easton, Penn. (1980).

Minor modifications of the NAC primary amino acid sequence may result in proteins which have substantially equivalent activity as compared to the NAC polypeptide described herein. Such modifications may be deliberate, as by site-directed mutagenesis, or may be spontaneous. All of the polypeptides produced by these modifications are included herein as long as the biological activity of NAC still exists. Further, deletion of one or more amino acids can also result in a modification of the structure of the resultant molecule without significantly altering its biological activity. This can lead to the development of a smaller active molecule which would have broader utility. For example, one can remove amino or carboxy terminal amino acids which may not be required for NAC biological activity.

The term "conservative variation" as used herein denotes the replacement of an amino acid residue by another, biologically similar residue. Examples of conservative variations include the substitution of one hydrophobic residue such as isoleucine, valine, leucine or methionine for another, or the substitution of one polar residue for another, such as the substitution of arginine for lysine, glutamic for aspartic acids, or glutamine for asparagine, and the like. The term "conservative variation" also includes the use of a substituted amino acid in place of an unsubstituted parent amino acid provided that antibodies raised to the substituted polypeptide also immunoreact with the unsubstituted polypeptide.

By "functional derivative" is meant the "fragments," "variants," "analogues," or "chemical derivatives" of a molecule. A "fragment" of a molecule, such as any of the DNA sequences of the present invention, includes any nucleotide subset of the molecule. A "variant" of such molecule refers to a naturally occurring molecule substantially similar to either the entire molecule, or a fragment

thereof. An "analog" of a molecule refers to a non-natural molecule substantially similar to either the entire molecule or a fragment thereof.

Similarly, a "functional derivative" of a gene encoding NACP polypeptide of the present invention includes "fragments", "variants", or "analogues" of the gene, including degenerate polynucleotides whose sequence may be determined readily by one of ordinary skill in the art, which encode a molecule possessing similar activity to a NAC peptide or fragment thereof.

Thus, as used herein, NAC or NACP polypeptide and NAC or NACP polynucleotide, include any functional derivative, fragments, variants, analogues, chemical derivatives which may be substantially similar to the NAC polypeptides and polynucleotides described herein and which possess similar activity.

Peptides of the invention can be synthesized by the well known solid phase peptide synthesis methods described Merrifield, *J. Am. Chem. Soc.*, 85:2149, (1962), and Stewart and Young, *Solid Phase Peptides Synthesis*, (Freeman, San Francisco, 1969, pp.27-62), using a copoly(styrene-divinylbenzene) containing 0.1-1.0 mMol amines/g polymer. On completion of chemical synthesis, the peptides can be deprotected and cleaved from the polymer by treatment with liquid HF-10% anisole for about 1/4-1 hours at 0°C. After evaporation of the reagents, the peptides are extracted from the polymer with 1% acetic acid solution which is then lyophilized to yield the crude material. This can normally be purified by such techniques as gel filtration on Sephadex G-15 using 5% acetic acid as a solvent. Lyophilization of appropriate fractions of the column will yield the homogeneous peptide or peptide derivatives, which can then be characterized by such standard techniques as amino acid

analysis, thin layer chromatography, high performance liquid chromatography, ultraviolet absorption spectroscopy, molar rotation, solubility, and quantitated by the solid phase Edman degradation.

As used herein, the terms "polynucleotide" or "NACP polynucleotide" denotes DNA, cDNA and RNA which encode NACP polypeptide as well as untranslated sequences which flank the structural gene encoding NACP. It is understood that all polynucleotides encoding all or a portion of NACP polypeptide of the invention, such as the NAC polypeptide(s) are also included herein, as long as the encoded polypeptide exhibits the activity or function of NACP or the tissue expression pattern characteristic of NACP. Such polynucleotides include naturally occurring forms, such as allelic variants, and intentionally manipulated forms, for example, mutagenized polynucleotides, as well as artificially synthesized polynucleotides. Such mutagenized polynucleotides can be produced, for example, by subjecting NAC or NACP polynucleotide to site-directed mutagenesis.

As described above, in another embodiment, a polynucleotide of the invention also includes in addition to NACP and/or NAC coding regions, those nucleotides which flank the coding region of the NACP structural gene. For example, a polynucleotide of the invention includes 5' regulatory nucleotide sequences and 3' untranslated sequences associated with the NACP structural gene.

The polynucleotide sequence for NACP also includes antisense sequences. The polynucleotides of the invention also include sequences that are degenerate as a result of the genetic code. There are 20 natural amino acids, most of which are specified by more than one codon. Therefore, as long as

the amino acid sequence of NACP results in a functional polypeptide (at least, in the case of the sense polynucleotide strand), all degenerate nucleotide sequences are included in the invention. Where the antisense polynucleotide is concerned, the invention embraces all antisense polynucleotides capable of inhibiting production of NACP polypeptide.

The preferred NACP cDNA clone of the invention is defined by a sequence of 1560 basepairs, in accordance with the transcript of 1.6 kb. A minor transcript of 3.6 kb is also found. The sequence surrounding the predicted initiator methionine codon (GCCATGG) agrees with the Kozak consensus sequence according to K. Kozak (*Nucleic Acids Res.*, 15:8125-8148, 1987). The nearest in-frame stop codon is found 18 bp upstream of the ATG initiation codon. As shown in FIGURE 2A, the nucleotide sequences encoding the X- and Y-peptide sequences are localized contiguously in the middle of the precursor peptide at bp 233 to 337. The preferred NACP cDNA clone is characterized by the lack of a sequence encoding a signal peptide and by the lack of N-linked glycosylation sites.

DNA sequences of the invention can be obtained by several methods. For example, the DNA can be isolated using hybridization procedures which are well known in the art. These include, but are not limited to : 1) hybridization of probes to genomic or cDNA libraries to detect shared nucleotide sequences; 2) antibody screening of expression libraries to detect shared structural features and 3) synthesis by the polymerase chain reaction (PCR).

Hybridization procedures are useful for the screening of recombinant clones by using labeled mixed synthetic oligonucleotide probes where each probe is potentially the complete complement of a specific DNA sequence in the

hybridization sample which includes a heterogeneous mixture of denatured double-stranded DNA. For such screening, hybridization is preferably performed on either single-stranded DNA or denatured double-stranded DNA. Hybridization is particularly useful in the detection of cDNA clones derived from sources where an extremely low amount of mRNA sequences relating to the polypeptide of interest are present. In other words, by using stringent hybridization conditions directed to avoid non-specific binding, it is possible, for example, to allow the autoradiographic visualization of a specific cDNA clone by the hybridization of the target DNA to that single probe in the mixture which is its complete complement (Wallace, *et al., Nucleic Acid Research*, 9:879, 1981).

A NACP containing cDNA library can be screened by injecting the various mRNA derived from cDNAs into oocytes, allowing sufficient time for expression of the cDNA gene products to occur, and testing for the presence of the desired cDNA expression product, for example, by using antibody specific for NACP or the X or Y peptide fragments thereof polypeptide or by using probes for the repeat motifs and a tissue expression pattern characteristic of NACP. Alternatively, a cDNA library can be screened indirectly for NACP polypeptides having at least one epitope using antibodies specific for the polypeptides, such as X and Y peptides. Such antibodies can be either polyclonally or monoclonally derived and used to detect expression product indicative of the presence of NACP cDNA.

Screening procedures which rely on nucleic acid hybridization make it possible to isolate any gene sequence from any organism, provided the appropriate probe is available. Oligonucleotide probes, which correspond to a part of the sequence encoding the protein in question, can be synthesized chemically.

This requires that short, oligopeptide stretches of amino acid sequence must be known. The DNA sequence encoding the protein can be deduced from the genetic code, however, the degeneracy of the code must be taken into account. It is possible to perform a mixed addition reaction when the sequence is degenerate. This includes a heterogeneous mixture of denatured double-stranded DNA.

For such screening, hybridization is preferably performed on either single-stranded DNA or denatured double-stranded DNA. Hybridization is particularly useful in the detection of cDNA clones derived from sources where an extremely low amount of mRNA sequences relating to the polypeptide of interest are present. In other words, by using stringent hybridization conditions directed to avoid non-specific binding, it is possible, for example, to allow the autoradiographic visualization of a specific cDNA clone by the hybridization of the target DNA to that single probe in the mixture which is its complete complement (Wallace, *et al., Nucl. Acid Res.*, 9:879, 1981).

The development of specific DNA sequences encoding NACP, or fragments thereof, can also be obtained by: 1) isolation of double-stranded DNA sequences from the genomic DNA; 2) chemical manufacture of a DNA sequence to provide the necessary codons for the polypeptide of interest; and 3) *in vitro* synthesis of a double-stranded DNA sequence by reverse transcription of mRNA isolated from a eukaryotic donor cell. In the latter case, a double-stranded DNA complement of mRNA is eventually formed which is generally referred to as cDNA. Of these three methods for developing specific DNA sequences for use in recombinant procedures, the isolation of genomic DNA isolates is the least common. This is especially true when it is desirable

to obtain the microbial expression of mammalian polypeptides due to the presence of introns.

The synthesis of DNA sequences is frequently the method of choice when the entire sequence of amino acid residues of the desired polypeptide product is known. When the entire sequence of amino acid residues of the desired polypeptide is not known, the direct synthesis of DNA sequences is not possible and the method of choice is the synthesis of cDNA sequences. Among the standard procedures for isolating cDNA sequences of interest is the formation of plasmid- or phage-carrying cDNA libraries which are derived from reverse transcription of mRNA which is abundant in donor cells that have a high level of genetic expression. When used in combination with polymerase chain reaction technology, even rare expression products can be cloned. In those cases where significant portions of the amino acid sequence of the polypeptide are known, the production of labeled single or double-stranded DNA or RNA probe sequences duplicating a sequence putatively present in the target cDNA may be employed in DNA/DNA hybridization procedures which are carried out on cloned copies of the cDNA which have been denatured into a single-stranded form (Jay, *et al., Nucl. Acid Res.*, 11:2325, 1983).

A cDNA expression library, such as lambda gt11, can be screened indirectly for NACP or NAC peptides having at least one epitope, using antibodies specific for NACP or the NAC peptide. Such antibodies can be either polyclonally or monoclonally derived and used to detect expression product indicative of the presence of NACP cDNA.

DNA sequences encoding NACP or NAC can be expressed *in vitro* by DNA transfer into a suitable host cell. "Host cells" are cells in which a vector can be propagated and its DNA expressed. The term also includes any progeny of the subject host cell. It is understood that all progeny may not be identical to the parental cell since there may be mutations that occur during replication. However, such progeny are included when the term "host cell" is used. Methods of stable transfer, in other words when the foreign DNA is continuously maintained in the host, are known in the art.

In the present invention, the NACP or NAC polynucleotide sequences may be inserted into a recombinant expression vector. The term "recombinant expression vector" refers to a plasmid, virus or other vehicle known in the art that has been manipulated by insertion or incorporation of the NACP genetic sequences. Such expression vectors contain a promoter sequence which facilitates the efficient transcription of the inserted genetic sequence of the host. The expression vector typically contains an origin of replication, a promoter, as well as specific genes which allow phenotypic selection of the transformed cells. Vectors suitable for use in the present invention include, but are not limited to the T7-based expression vector for expression in bacteria (Rosenberg, *et al., Gene*, 56:125, 1987), the pMSXND expression vector for expression in mammalian cells (Lee and Nathans, *J. Biol. Chem.*, 263:3521, 1988) and baculovirus-derived vectors for expression in insect cells. The DNA segment can be present in the vector operably linked to regulatory elements, for example, a promoter (e.g., T7, metallothionein I, or polyhedrin promoters and enhancer).

Polynucleotide sequences encoding NACP or NAC peptides can be expressed in either prokaryotes or eukaryotes. Hosts can include microbial, yeast, insect and mammalian organisms. Methods of expressing DNA sequences having eukaryotic or viral sequences in prokaryotes are well known in the art. Biologically functional viral and plasmid DNA vectors capable of expression and replication in a host are known in the art. Such vectors are used to incorporate DNA sequences of the invention.

Transformation of a host cell with recombinant DNA may be carried out by conventional techniques as are well known to those skilled in the art. Where the host is prokaryotic, such as *E. coli*, competent cells which are capable of DNA uptake can be prepared from cells harvested after exponential growth phase and subsequently treated by the $CaCl_2$ method by procedures well known in the art. Alternatively, $MgCl_2$ or RbCl can be used. Transformation can also be performed after forming a protoplast of the host cell or by electroporation.

When the host is a eukaryote, such methods of transfection of DNA as calcium phosphate co-precipitates, conventional mechanical procedures such as microinjection, electroporation, insertion of a plasmid encased in liposomes, or virus vectors may be used. Eukaryotic cells can also be cotransformed with DNA sequences encoding the NACP or NAC proteins of the invention, and a second foreign DNA molecule encoding a selectable phenotype, such as the herpes simplex thymidine kinase gene. Another method is to use a eukaryotic viral vector, such as simian virus 40 (SV40) or bovine papilloma virus, to transiently infect or transform eukaryotic cells and express the protein. (*Eukaryotic Viral Vectors*, Cold Spring Harbor Laboratory, Gluzman ed., 1982).

Isolation and purification of microbially expressed polypeptide, or fragments thereof, provided by the invention, may be carried out by conventional means including preparative chromatography and immunological separations involving monoclonal or polyclonal antibodies.

One skilled in the art will also be able to create a living mouse model for expressing the NACP gene in a living transgenic mouse. Methods of making a transgenic mouse expressing a foreign gene utilize several techniques for inserting the foreign gene into the germline of the animal at an early developmental stage, such as at the single-cell level. For instance, the transgene can be inserted into a mouse oocyte, which is then implanted into mouse for birth of a transgenic animal. See U. S. Patent No. 4,873,191, "Genetic Transformaion of Zygotes," which is incorporated herein in its entirety. Similarly, pluripotent embroyo-derived stem (ES) cells, can be modified extra-corporeally by insertion of a cloned gene to transfer a modification to the germ line of a living organism.

Homologous recombination has also been used for targeting genetic mutations to a predetermined genetic locus of an ES cell in order to produce a transgenic animal (Mansour, *et al.*, *Nature*, 336:348, 1988; Capecchi, M., *Trends Genet.*, 5:70, 1989). Homologous recombination between DNA sequences residing in the chromosome and newly introduced cloned DNA sequences allows the transfer of any modification to the cloned gene into the genome of a living cell. Several site-specific recombination systems are known (Craig, *Ann. Rev. Genet.*, 22:77, 1988) including the FLP system of yeast and the Cre system of bacteriophage P1. The FLP recombinase of the yeast *saccharomyces cerevisiae* acts on copies of a recombination target called FRTs. The FLP system has been shown to effect site-specific recombination

in the *Drosophilia* genome *in vivo* (Golic, *et al., Cell*, 59:499, 1989, Golic, K., *Science*, 252:958, 1991) and in eukaryotic cells *in vitro* (O'Gorman, *et al., Science*, 251:1351, 1991).

A novel approach to effecting specific homologous recombination events in eukaryotes is the prokaryotic Cre-loxP site-specific DNA recombination system of coliphage P1. The 38 kD Cre protein efficiently causes both inter- and intra-molecular recombination between specific 34 base pair repeats termed loxP (Sternberg, *et al., J. Mol. Biol.*, 150:467, 1981). Each loxP site contains two 13 base pair inverted repeats and an 8 base pair asymmetric core sequence. No accessory proteins are required for exchange to occur. Direct repeats of loxP dictate an excision of intervening sequences while inverted repeats specify inversion. Cre has been shown to be functional in eukaryotic cells (Sauer, *et al., Nucleic Acids Res.*, 27:147, 1989) and in transgenic plants (Dale, *et al., Proc. Natl. Acad. Sci. USA*, 88:10558, 1991).

These and other types of "gene targeting" provide a means for controlling the site of integration (Smithies, *et al., Nature*, 317:230, 1985). For homologous recombination to occur between two DNA molecules, the molecules must possess a region of sequence identity with respect to one another, typically several hundred base pairs in length. This method requires that the gene of interest must have been previously cloned, and the intron-exon boundaries determined, as is the case herein. Targeted insertion increases the probability that an inserted gene will function as desired. It also reduces the chance of random insertion activating a quiescent oncogene or inactivating a cancer supressor gene.

U. S. Patent Nos. 5,175,383, 5,175,381 and 5,175,385, which are incorporated herein by reference in their entirety, illustrate utilization of these methods of targeted insertion to either correct or mutate a desired chromosomal locus, thereby creating a transgenic animal. U. S. Patent No. 5,175,383 discloses the method of making a transgenic mouse model for the human disease benign prostatic hypertrophy. To create the Harvard mouse the int-2 oncogene, which codes for a growth factor, was joined to a control gene to ensure that the growth factor would be produced in prostate tissue. Systems for studying regulation of genes in transgenic animal are also disclosed in Patent Application WO 90/06367 entitled "Transgenic Mice for the Analysis of Hair Growth" wherein insertion of a promoter of a gene for a hair specific protein, one expressed only in tissues involved in hair growth, is used to regulate expression of a reporter gene. Due to conservation among homologous genes and their products, transgenes can be expressed in mice under the control of a regulatory sequence from a human tissue specific gene. Recently, Patent Application WO 93/14200, which is incorporated herein by reference in its entirety, discloses creation of a trangenic mouse that expresses β-amyloid precursor proteins.

D. METHODS FOR USE OF NAC ANTISENSE POLYNUCLEOTIDES

The NAC polynucleotide in the form of an antisense polynucleotide is useful in treating disease states associated with formation of amyloid i.e., amyloidosis in the brain, (particularly in neuritic) plaques by preventing expression of the protein that is originating. Essentially, any disorder which is etiologically linked to expression of NACP could be considered susceptible to treatment with a reagent of the invention which modulates NACP expression. The term "modulate" envisions the suppression of expression of NACP when it is

over-expressed, or augmentation of NACP expression when it is under-expressed or when the NACP expressed is a mutant form of the polypeptide.

When amyloidosis is associated with NACP overexpression, such suppressive reagents as antisense NACP polynucleotide sequence or NACP binding antibody can be introduced to a cell. Alternatively, when an amyloid disorder is associated with underexpression or expression of a mutant NACP polypeptide, a sense polynucleotide sequence (the DNA coding strand) or NACP polypeptide can be introduced into the cell. Methods for use of antisense gene therapy are discussed in greater detail below.

E. ANTI-NAC AND ANTI-NACP ANTIBODIES.

The invention includes polyclonal and monoclonal antibodies immunoreactive with NACP or NAC polypeptides or immunogenic fragments thereof.

Antibodies which are specific for NAC or NACP may be produced by immunization of a non-human with antigenic NAC or NACP peptides of native or synthetic origin. Once antigenic peptides are prepared, antibodies to the immunizing peptide are produced by introducing peptide into a mammal (such as a rabbit, mouse or rat).

A multiple injection immunization protocol is preferred for use in immunizing animals with the antigenic MTA peptides (see, e.g., Langone, *et al.*, eds., "Production of Antisera with Small Doses of Immunogen: Multiple Intradermal Injections", *Methods of Enzymology* (Acad. Press, 1981). For example, a good antibody response can be obtained in rabbits by intradermal injection of 1 mg

of the antigenic MTA peptide emulsified in Complete Freund's Adjuvant followed several weeks later by one or more boosts of the same antigen in Incomplete Freund's Adjuvant.

If desired, the immunizing peptide may be coupled to a carrier protein by conjugation using techniques which are well-known in the art. Such commonly used carriers which are chemically coupled to the peptide include keyhole limpet hemocyanin (KLH), thyroglobulin, bovine serum albumin (BSA), and tetanus toxoid. The coupled peptide is then used to immunize the animal (e.g. a mouse or a rabbit).

Polyclonal antibodies produced by the animals can be further purified, for example, by binding to and elution from a matrix to which the peptide to which the antibodies were raised is bound. Those of skill in the art will know of various techniques common in the immunology arts for purification and/or concentration of polyclonal antibodies, as well as monoclonal antibodies (see, for example, Coligan, *et al.*, Unit 9, *Current Protocols in Immunology,* Wiley Interscience, 1991).

If desired, polyclonal antibodies can be further purified, for example, by binding to and elution from a matrix to which NAC polypeptide is bound. Those of skill in the art will know of various other techniques common in the immunology arts for purification and/or concentration of polyclonal antibodies, as well as monoclonal antibodies.

Antibodies which consist essentially of pooled monoclonal antibodies with different epitopic specificities, as well as distinct monoclonal antibody preparations are provided. Monoclonal antibodies are made from antigen

containing fragments of the protein by methods well known to those skilled in the art (Kohler, *et al., Nature,* 256:495, 1975). The term antibody or, immunoglobulin as used in this invention includes intact molecules as well as genetically engineered antibody constructs such as bifunctional antibodies, CDR grafted antibodies, and the like, as well as fragments thereof, such as Fab and $F(ab')_2$, which are capable of binding an epitopic determinant on NACP or NAC.

A preferred method for the identification and isolation of an antibody binding domain that exhibits binding with NACP or NAC peptides is the bacteriophage λ vector system. This vector system has been used to express a combinatorial library of Fab fragments from the mouse antibody repertoire in *Escherichia coli* (Huse, *et al., Science,* 246:1275-1281, 1989) and from the human antibody repertoire (Mullinax, *et al., Proc. Natl. Acad. Sci.,* 87:8095-8099, 1990). As described therein, receptors (Fab molecules) exhibiting binding for a preselected ligand were identified and isolated from these antibody expression libraries. This methodology can also be applied to hybridoma cell lines expressing monoclonal antibodies with binding for a preselected ligand. Hybridomas which secrete a desired monoclonal antibody can be produced in various ways using techniques well understood by those having ordinary skill in the art and will not be repeated here. Details of these techniques are described in such references as *Monoclonal Antibodies-Hybridomas: A New Dimension in Biological Analysis,* Edited by Roger H. Kennett, *et al.*, Plenum Press, 1980; and, U.S. Patent No. 4,172,124.

F. METHODS FOR DETECTING AMYLOID OR A CELL EXPRESSING NACP.

The invention provides a method for detecting a cell expressing NACP, or an amyloid disorder associated with NAC, comprising contacting a cell suspected of expressing NACP or having a NAC associated disorder with a reagent which binds to the target component. The cell component can be nucleic acid, such as DNA or RNA, or protein. When the component is nucleic acid, the reagent is a nucleic acid probe or PCR primer. When the cell component is protein, the reagent is an antibody probe. The probes are detectably labeled, for example, with a radioisotope, a fluorescent compound, a bioluminescent compound, a chemiluminescent compound, a metal chelator or an enzyme. Those of ordinary skill in the art will know of other suitable labels for binding to the antibody or probe, or will be able to ascertain such, using routine experimentation.

For purposes of the invention, an antibody or nucleic acid probe specific for NACP or fragments thereof may be used to detect the presence of NACP polypeptide or NAC peptides (using antibody) or polynucleotide (using nucleic acid probe) in biological fluids or tissues. Any specimen containing a detectable amount of antigen can be used. A preferred sample in this invention is tissue of brain origin, specifically midfrontal cortex tissue obtained through biopsy. More preferably, the tissue is hippocampus tissue. Preferably the subject is human.

Another technique which may also result in greater sensitivity consists of coupling the antibodies to low molecular weight haptens. These haptens can then be specifically detected by means of a second reaction. For example, it

is common to use such haptens as biotin, which reacts with avidin, or dinitrophenyl, pyridoxal, and fluorescein, which can react with specific anti-hapten antibodies.

The method for detecting a cell expressing NACP or a amyloid disorder associated with NAC, described above, can be utilized for prescreening for detection of amyloidosis prior to or after a subject's manifestation of typical clinical and neuropathological features of AD. Additionally, the method for detecting NACP polypeptide in cells is useful for prescreening to detect risk of amyloid disorder by identifying cells expressing NACP at levels different than normal cells. Using the method of the invention, high, low, and mutant NACP expression can be identified in a cell and the appropriate course of treatment can be employed (e.g., sense or antisense gene therapy).

The monoclonal antibodies of the invention are suited for use, for example, in immunoassays in which they can be utilized in liquid phase or bound to a solid phase carrier. In addition, the monoclonal antibodies in these immunoassays can be detectably labeled in various ways. Examples of types of immunoassays which can utilize monoclonal antibodies of the invention are competitive and non-competitive immunoassays in either a direct or indirect format. Examples of such immunoassays are the radioimmunoassay (RIA) and the sandwich (immunometric) assay. Detection of the antigens using the monoclonal antibodies of the invention can be done utilizing immunoassays which are run in either the forward, reverse, or simultaneous modes, including immunohistochemical assays on physiological samples. Those of skill in the art will know, or can readily discern, other immunoassay formats without undue experimentation.

The monoclonal antibodies of the invention can be bound to many different carriers and used to detect the presence of NACP or NAC peptides such as X and Y. Examples of well-known carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, agaroses and magnetite. The nature of the carrier can be either soluble or insoluble for purposes of the invention. Those skilled in the art will know of other suitable carriers for binding monoclonal antibodies, or will be able to ascertain such using routine experimentation.

As used in this invention, the term "epitope" includes any determinant capable of specific interaction with the monoclonal antibodies of the invention. Epitopic determinants usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics.

The concentration of detectably labeled monoclonal antibody which is administered should be sufficient such that the binding to those cells having NAC or NACP is detectable compared to the background. Further, it is desirable that the detectably labeled monoclonal antibody be rapidly cleared from the circulatory system in order to give the best target-to-background signal ratio.

Because of the blood/brain barrier, it can be expected that antibodies will not be particularly the preferred reagant for use in *in vivo* applications. Rather, NAC/NACP polypeptides (particularly the former) that will cross the blood-brain barrier, and bind to the native protein are expected to be the best NAC/NACP ligands. In particular, the preferred ligands of the invention will be those which

are derived from positions 61-95 of the NACP amino acid sequence (i.e., in the NAC region), and from the C-terminal region of NACP (positions 131-140). In part, this preference is derived from the observation that antibodies to NAC (61-95) bind amyloid plaques, while antibodies to NACP (131-140) bind synapses, as well as the presumed ability of the peptides to cross the blood brain burner.

It has been shown that β-amyloid peptide fragments of about 28 amino acids in length or shorter will effectively cross the blood/brain barrier *in vivo* without toxic effect (see, e.g., Examples 15 and 16, as well as co-pending, commonly owned U.S. Patent Application No. 08/136,751; filed 10/14/93). Given the similarity in structure between the pleated β-amyloid molecule and NAC (see, Example 8, below), it can be expected that NAC peptides of about 28 amino acids or shorter in length would cross the blood-brain barrier. As shown in Example 8, NAC is a self-aggregating peptide (which apparently derives from the 61-95 amino acid region of NACP). Within the 61-95 stretch of amino acids (see, SEQ.ID.No.1), the following peptides have been determined to have self-aggregating ability (i.e., binding sites for NAC) using the method described in Example 8 (reading from the N to the C terminus):

TVEGAGSIAAATGFVKKD (NAC peptide 1)

and KKKTVEGAGSIAAATGFV (NAC peptide 2).

Further, the somewhat shorter NACP peptides described below (which are derived from the 131-140 region of NACP; see, SEQ.ID.No.1), would also be expected to cross the blood/brain barrier (reading from the N to the C terminus):

EGYQDYEPEAKKD (NACP peptide 1)

and KKKEGYQDYEPEA (NACP peptide 2).

Determination of whether a particular NAC or NACP peptide will specifically bind NAC or NACP can be readily made without undue experimentation by one of ordinary skill in the art. In regard to characterstics of peptides that may cross the blood/brain barrier, those of skill in the art may wish to refer to Pardridge, WA, "*Peptide Drug Delivery to the Brain*", (Raven Press, 1991), chapters 3, 6 and 7 of which in particular are incorporated herein by this reference to illustrate the state of knowledge in the art concerning delivery of peptides to the brain. An example of a suitable animal model and testing protocol for use in this regard are set forth in Examples 15-16.

As an illustration of techniques which may be employed to identify peptides that may cross the blood/brain barrier, without undue experimentation, peptides shorter than NAC (61-95) or NACP (131-140) can be screened for use in the method of the invention by incubation with AD brain tissue homogenates or brain tissue from an animal model which has been implanted surgically with amyloid, or through immunological techniques such as those described above (e.g., testing the reactivity of anti-NACP antibodies that react with the native protein to the candidate ligand).

It is also possible to determine without undue experimentation if a NAC or NACP peptide (i.e., NAC or NACP candidate ligand) has the same specificity as the NAC ligand described above by ascertaining whether the former prevents the latter from binding to NAC. If the candidate ligand competes with a ligand which is known to bind NAC/NACP (as shown by a decrease in binding by the latter), then the two peptides bind to the same, or a closely related site.

Still another way to determine whether a particular candidate ligand has the specificity of a ligand which is known to bind NAC/NACP is to preincubate the candidate ligand with NAC or NACP and then add a known ligand to determine if it is inhibited in its ability to bind NAC or NACP. If the known NAC/NACP ligand is inhibited, in all likelihood the candidate ligand has the same, or functionally equivalent, binding specificity as the known NAC/NACP ligand.

Screening of candidate ligands can also be determined by attaching a detectable label to them, incubating them with amyloid-containing brain tissue (*in vivo* or *in vitro*) and determining whether binding has occurred using *in vivo* diagnostic imaging techniques as described in more detail below.

NAC and NACP ligands may be labelled as described below; however, for *in vivo* diagnostic imaging, the use of radiolabels or paramagnetic isotopes will be preferred. For example, for *in vivo* diagnostic imaging, the type of detection instrument available is a major factor in selecting a given radioisotope. The radioisotope chosen must have a type of decay which is detectable for a given type of instrument. Still another important factor in selecting a radioisotope for *in vivo* diagnosis is that the half-life of the radioisotope be long enough so that it is still detectable at the time of maximum uptake by the target, but short enough so that deleterious radiation with respect to the host is minimized. Ideally, a radioisotope used for *in vivo* imaging will lack a particle emission, but produce a large number of photons in the 140-250 keV range, which may be readily detected by conventional gamma cameras.

For *in vivo* diagnosis, radioisotopes may be bound to proteinaceous ligands either directly or indirectly by using an intermediate functional group. Intermediate functional groups which often are used to bind radioisotopes

which exist as metallic ions to immunoglobulins are the bifunctional chelating agents such as diethylenetriaminepentacetic acid (DTPA) and ethylenediaminetetraacetic acid (EDTA) and similar molecules. Typical examples of metallic ions which can be bound to the ligands of the invention are ^{111}In, ^{97}Ru, ^{67}Ga, ^{68}Ga, ^{72}As, ^{89}Zr, and ^{201}Tl. However, for its relatively low toxicity and ready imaging, [TM^{99}] (pertechenetate) will be the most preferred radiolabel for its relatively low toxicity in mammals. Radiolabelling with ^{99m}Tc may be performed according to the technique described in Kasnia, *et al. J. Nucl. Med.*, 32:1445-1451, 1991.

However, for any *in vitro use*, ^{125}Iodide (^{125}I) would be preferred for ease of detection. ^{125}I may be attached to a NAC or NACP ligand for use in the invention by conventional techniques including oxidative radiodination using sodium ^{125}I and chloramine T (for tyrosine containing peptides) or the acylation followed by oxidative radiodination (for peptides not containing tyrosine). Iodination may also be performed using an iodination product from DuPont of Wilmington, DE (marketed under the trademark NEN) or the iodogen technique described in Salacinski, *et al.*, *Anal. Biochem.*, 117:136-146, 1981. Iodogen for use in this method is commercially available from Pierce and Wariner, Chester, England.

The ligands of the invention can also be labeled with a paramagnetic isotope for purposes of *in vivo* diagnosis, as in magnetic resonance imaging (MRI) or electron spin resonance (ESR). In general, any conventional method for visualizing diagnostic imaging can be utilized. Usually gamma and positron emitting radioisotopes are used for camera imaging and paramagnetic isotopes for MRI. Elements which are particularly useful in such techniques include ^{157}Gd, ^{55}Mn, ^{162}Dy, ^{52}Cr, and ^{56}Fe.

Regardless of the detectable label used, the labelled ligands will preferably be purified by means well known in the art referred to above (for example, RP-HPLC) to an essentially quantitative specific activity (e.g., about 2000 Ci/mmol; 1Ci≈ 37GBq).

To practice the invention, a diagnostically effective amount of a detectably labelled ligand as described above will be administered to mammal which is suspected of having AD, has been diagnosed as having AD or, in the research context, has had amyloid plaque formation induced in its brain tissue. In the preferred embodiment, the mammal will be a human who is suspected of having or has been diagnosed as having AD.

As a rule, the dosage of detectably labeled ligand for *in vivo* diagnosis will vary depending on such factors as age, sex, and extent of disease of the individual. The dosage of monoclonal antibody can vary from about 0.001 mg/m^2 to about 500 mg/m^2, preferably 0.1 mg/m^2 to about 200 mg/m^2, most preferably about 0.1 mg/m^2 to about 10 mg/m^2. Such dosages may vary, for example, depending on whether multiple injections are given, amyloid burden, and other factors known to those of skill in the art.

Those skilled in the art will be able to determine an appropriate dosage for the detectably labelled ligands based on the animal study data provided in the examples below. In general, the "diagnostically effective amount" of detectably labelled NAC ligand for *in vivo* applicants will be that amount which is sufficient to detectibly bind any NAC present in the subject brain tissue.

Although binding of the detectably labelled peptides of the invention is somewhat dose-dependent, it will be appreciated because the peptides are self-aggregating, increasing their dosage may intensify rather than expand the NAC binding pattern. More specifically, while binding of most or all of the NAC plaques present in the subject brain tissue may occur at lower dosage levels, the intensity of the emissions indicative of that binding may be enhanced as the detectably labelled peptide density per plaque is increased at higher dosage levels.

Although any nonsurgical route of administration which introduces the detectably labelled ligands of the invention into brain tissue may be used, intraarterial injection in preferred, with intracarotid injections being most preferred. Where the method of the invention is being performed for diagnostic purposes, the background measurement will preferably be established by determining the extent of binding of a detectably labelled ligand in healthy mammalian subjects. In this context, "healthy" is defined as a mammal with less than about 15 amyloid plaques/unit area of brain tissue (one unit area = 0.1 square millimeter) and/or a subject who exhibits no clinical signs of a neuropsychological disorder. In the same regard, measurements based on binding of the detectably labelled ligand indicative of the presence of ≥ about 15 plaques/unit area will be considered to be diagnostically significant for (i.e., indicative of) AD. These data can be used to assist in confirmation or refutation of a clinical diagnosis of AD.

To evaluate the prognosis of a subject who is suspected of having or has been diagnosed as having AD, the method of the invention can provide data of at least three significant types. First, using NAC ligand, plaque density in excess of about 15 plaques/unit area can be correlated to the progress of the disease

(i.e., greater plaque density would be expected to be present in later stages of the disease). Second, measurements of plaque development taken over time will be indicative of the rate at which the disease is progressing and/or of the effectiveness of a particular treatment or therapy. In applying the inventive method to evaluate the progression of the disease, previous plaque density measurements taken from that subject would be used as background. Third, using NACP ligand, the probable progression of loss of cognitive function as well as the status of plaque formation through NACP cleavage, may be evaluated based on decreses in the synaptic population and/or increases in presynatpic bouton concentrations of NACP.

Binding will preferably be measured within one hour of introducing the detectably labelled peptide into the subject's bloodstream. Binding will be measured *in vivo* using well-known *in vivo* diagnostic imaging techniques (in particular computer assisted sectional radiography (tomography)), preferably during the first hour following administration of the detectably labelled peptide.

Of the presently known tomography techniques, positron emission tomography (PET) and single photon emission computed tomography (SPECT) are preferred for use in the method of the invention. Because the appropriate use of these techniques will be known or apparent to those skilled in the art, their use will not be described in detail here.

For both prognosis and diagnosis, it may be desirable to evaluate the results of the *in vivo* binding assay of the invention in combination with evidence of the synaptic integrity of the subject brain tissue as well as clinical signs of disease. One suitable *in vitro* technique for evaluating and detecting synaptic loss in sections of brain tissue using anti-synaptophysin antibodies is described in

Masliah, *et al., Am. J. Pathol.,* 137:1293-1297 (1990), the disclosure of which is hereby incorporated by reference to demonstrate techniques for evaluating synaptic loss known in the art; other techniques will be known to those of skill in the neurological arts.

For research purposes, the NAC/NACP ligands may be used for *in vitro* studies, for example, binding affinity of different peptides, to develop anti-amyloid antibodies, to study the pathology of amyloid deposition and to evaluate proposed therapies. The NAC/NACP peptides may be of particular use in developing *in vivo* means of differential diagnosis. For example, because NAC principally appears in mature plaques, the stage of AD development is a given patient may be identified more accurately by detecting NAC *in vivo* than is now possible using conventional diagnostic techniques.

More generally, the NAC/NACP ligands of the invention can be used to monitor the course of amelioration of NAC associated amyloid disorder. Thus, by measuring the increase or decrease in the number of cells expressing NACP or changes in the concentration of normal versus mutant NACP or NAC present in various body fluids and/or tissues, it would be possible to determine whether a particular therapeutic regiment aimed at ameliorating the disorder is effective.

G. THERAPEUTIC METHODS FOR TREATING NAC ASSOCIATED AMYLOID DISORDER.

The present invention also provides a method for treating a subject with a NAC associated amyloid disorder. Because the NACP nucleotide sequence can be expressed in an altered manner as compared to expression in a normal cell,

it is possible to design appropriate therapeutic or diagnostic techniques directed to this sequence. Thus, where an amyloid disorder is associated with the over-expression of NACP, nucleic acid sequences that interfere with NACP expression at the translational level can be used. This approach utilizes, for example, antisense nucleic acid and ribozymes to block translation of a specific NACP mRNA, either by masking that mRNA with an antisense nucleic acid or by cleaving it with a ribozyme. In cases when a amyloid disorder or abnormal cell phenotype is associated with the under expression of NACP or expression of a mutant NACP polypeptide, nucleic acid sequences encoding NACP (sense) could be administered to the subject with the disorder.

Further, as indicated in the Background of the Invention, studies regarding accumulation of β-amyloid in brain tissue have indicated that binding of synthetic β-amyloid peptide to native β-amyloid actually retards the accumulation of the native protein. Based on these results, and given the physical and functional relationships between NAC and β-amyloid (see, Examples 12 and 13 below), it can be expected that administration of NAC peptides will provide a therapeutic benefit to a person suffering from a NAC associated amyloid disorder, such as AD. NAC peptides that will cross the blood-brain barrier and bind to NAC (to "modulate" the accumulation thereof) are identified elsewhere above, as are means to identify any additional NAC peptides possessing this ability.

Antisense nucleic acids are DNA or RNA molecules that are complementary to at least a portion of a specific mRNA molecule (Weintraub, *Scientific American*, 262:40, 1990). In the cell, the antisense nucleic acids hybridize to the corresponding mRNA, forming a double-stranded molecule. The antisense nucleic acids interfere with the translation of the mRNA since the cell will not

translate a mRNA that is double-stranded. Antisense oligomers of about 15 nucleotides are preferred, since they are easily synthesized and are less likely to cause problems than larger molecules when introduced into the target NACP-producing cell. The use of antisense methods to inhibit the *in vitro* translation of genes is well known in the art (Marcus-Sakura, *Anal. Biochem.*, 172:289, 1988).

Ribozymes are RNA molecules possessing the ability to specifically cleave other single-stranded RNA in a manner analogous to DNA restriction endonucleases. Through the modification of nucleotide sequences which encode these RNAs, it is possible to engineer molecules that recognize specific nucleotide sequences in an RNA molecule and cleave it (Cech, *J. Amer. Med. Assn.*, 260:3030, 1988). A major advantage of this approach is that, because they are sequence-specific, only mRNAs with particular sequences are inactivated.

There are two basic types of ribozymes namely, *tetrahymena*-type (Hasselhoff, *Nature*, 334:585, 1988) and "hammerhead"-type. *Tetrahymena*-type ribozymes recognize sequences which are four bases in length, while "hammerhead"-type ribozymes recognize base sequences 11-18 bases in length. The longer the recognition sequence, the greater the likelihood that that sequence will occur exclusively in the target mRNA species. Consequently, hammerhead-type ribozymes are preferable to *tetrahymena*-type ribozymes for inactivating a specific mRNA species and 18-based recognition sequences are preferable to shorter recognition sequences.

The present invention also provides gene therapy for the treatment of amyloid disorders which are mediated by NACP protein. Such therapy would achieve its therapeutic effect by introduction of the NACP antisense polynucleotide, into target cells (i.e., in brain tissue) of subjects having the amyloid disorder. Delivery of antisense NACP polynucleotide can be achieved using a recombinant expression vector such as a chimeric virus or a colloidal dispersion system. Disorders associated with under-expression of NACP could similarly be treated using gene therapy with sense nucleotide sequences.

Various viral vectors which can be utilized for gene therapy as taught herein include adenovirus, herpes virus, vaccinia, or, preferably, an RNA virus such as a retrovirus. Preferably, the retroviral vector is a derivative of a murine or avian retrovirus. Examples of retroviral vectors in which a single foreign gene can be inserted include, but are not limited to: Moloney murine leukemia virus (MoMuLV), Harvey murine sarcoma virus (HaMuSV), murine mammary tumor virus (MuMTV), and Rous Sarcoma Virus (RSV). A number of additional retroviral vectors can incorporate multiple genes. All of these vectors can transfer or incorporate a gene for a selectable marker so that transduced cells can be identified and generated. By inserting a NACP sequence of interest into the viral vector, along with another gene which encodes the ligand for a receptor on a specific target cell, for example, the vector is now target specific. Retroviral vectors can be made target specific by inserting, for example, a polynucleotide encoding an enzyme that determines the structure of a sugar, a glycolipid, or a protein. Preferred targeting is accomplished by using an antibody to target the retroviral vector. Those of skill in the art will know of, or can readily ascertain without undue experimentation, specific polynucleotide sequences which can be inserted into the retroviral genome to allow target

specific delivery of the retroviral vector containing the NACP antisense polynucleotide.

Since recombinant retroviruses are defective, they require assistance in order to produce infectious vector particles. This assistance can be provided, for example, by using helper cell lines that contain plasmids encoding all of the structural genes of the retrovirus under the control of regulatory sequences within the LTR. These plasmids are missing a nucleotide sequence that enables the packaging mechanism to recognize an RNA transcript for encapsidation. Helper cell lines which have deletions of the packaging signal include but are not limited to ψ2, PA317 and PA12, for example. These cell lines produce empty virions, since no genome is packaged. If a retroviral vector is introduced into such cells in which the packaging signal is intact, but the structural genes are replaced by other genes of interest, the vector can be packaged and vector virion produced.

Alternatively, NIH 3T3 or other tissue culture cells can be directly transfected with plasmids encoding the retroviral structural genes *gag, pol* and *env*, by conventional calcium phosphate transfection. These cells are then transfected with the vector plasmid containing the genes of interest. The resulting cells release the retroviral vector into the culture medium.

Another targeted delivery system for NACP antisense polynucleotides is a colloidal dispersion system. Colloidal dispersion systems include macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. The preferred colloidal system of this invention is a liposome. Liposomes are artificial membrane vesicles which are useful as delivery vehicles